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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
C12N 15/00, 1/20, C12P 21/02
C07K 13/00, A61K 37/02

A1

(11) International Publication Number: WO 88/ 0316

(43) International Publication Date: 5 May 1988 (05.05.88

(21) International Application Number: PCT/EP87/00621

(22) International Filing Date: 21 October 1987 (21.10.87)

(31) Priority Application Number: 8625435

(32) Priority Date: 23 October 1986 (23.10.86)

(33) Priority Country: GB

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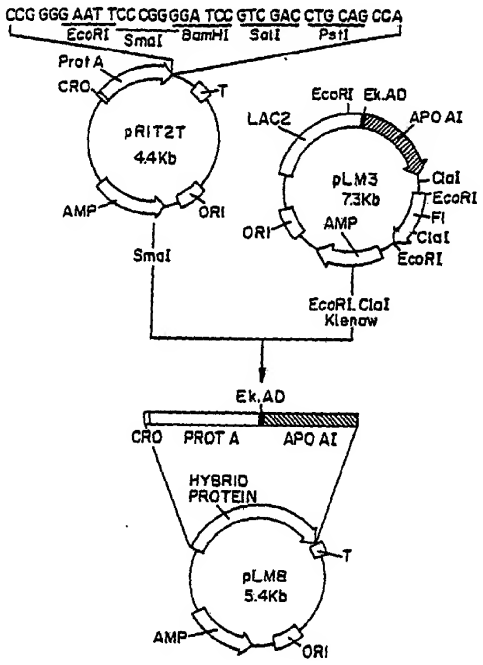
(81) Designated States: AU, DK, FI, HU, JP, KR, NO, SU.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receip of amendments.

(54) Title: HUMAN APOLIPOPROTEIN AI AND VARIANT FORM OF THE SAME EXPRESSED IN ESCHERICHIA COLI

(57) Abstract

An expression vector capable of expressing, in a transformed host, a protein which is capable of being detected by ELISA with anti-human apo-AI antiserum and which has the formula Met-X-Y, wherein X is a bond, a carrier peptide sequence which comprises a sequence derived from the N-terminal amino acid residues of beta-galactosidase or a sequence comprising one or more IgG-binding domains of Protein A, or the pro sequence of human apoAI; and Y represents the sequence of human apoAI or a genetic variant thereof. Hosts transformed with such vectors produce the protein, which is useful in the treatment of atherosclerosis and cardio-vascular diseases.



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DESCRIPTION

"HUMAN APOLIPOPROTEIN AI AND VARIANT FORM OF THE SAME EXPRESSED IN ESCHERICHIA COLI"

The present invention relates to the production of human apolipoprotein AI (apoAI) and its variants by means of recombinant DNA technology.

Atherosclerosis, and its attendant complications (i.e. coronary heart disease, CHD), is one and perhaps the most critical health problem in the world. A great number of risk factors have been associated with the development of the disease, one of the most important of these being elevated plasma cholesterol (CHL) levels. Thus, a great deal of attention has been devoted to the study of CHL metabolism in man.

The lipoprotein transport system holds the key to understanding the mechanisms by which genes, diet and hormones interact to regulate the plasma CHL and triglyceride (TG) levels in man. The function of the lipoproteins in plasma is mainly to transport lipids from one organ to another. It has recently become evident that they not only solubilize hydrophobic lipids, but may also dictate the site of the body to which each lipid class is to be delivered. There are four main classes of lipoproteins: chylomicrons (CM), very low density (VLDL), low density (LDL) and high density (HDL) lipoproteins.

For transport in plasma, TG and cholesteryl esters (CHLE) are packaged into lipoprotein particles in which they form a hydrophobic core surrounded by a surface monolayer of polar phospholipids (PL). The surface coat also contains unesterified CHL in relatively small amounts together with proteins called apolipoproteins (apo). At least nine apos have been identified: AI, AII, AIV, B (48-100), CI, CII, CIII, D and E.

Of particular interest is the investigation on

the relations between plasma lipoprotein levels and the risk of development of CHD. Both HDL and LDL are carriers of CHL and CHLE; however there are indications that, while LDL-CHL levels are a positive risk factor (Kannel et al., Ann. Intern. Med., 90:85-91, 1979), HDL's are an important negative risk factor (Yaari et al., Lancet, i:1011-1015, 1981). Although the precise function and mode of action of these lipoproteins has not yet been completely understood, it appears that HDL serves particularly to remove CHL from peripheral tissues and transport it back to the liver, with a mechanism named reverse CHL transport (RCT).

The chief apolipoprotein of HDL is apoAI and several studies have shown an inverse association between plasma apoAI levels and CHD similar to that which has been documented for HDL-CHL levels and CHD (Ishikawa et al., Eur. J. Clin. Invest., 8:179-182, 1978). In addition, both HDL-CHL and apoAI levels are inversely correlated to the severity to angiographically demonstrable coronary arteriosclerotic lesions (Pearson et al., Amer. J. Epidem., 109:285-295, 1979; Maciejko et al., New Eng. J. Med., 309:385-389, 1983). Thus it can be argued that high concentrations of HDL in plasma influence CHD by retarding atherogenesis and/or promoting regression of existing lesions.

Complexes of HDL apolipoproteins, mainly apoAI, and lecithin promote the efflux of free CHL from cells in vitro, including cultured arterial smooth muscle cells (Stein et al., Biochem. Biophys. Acta, 380:106-118, 1975). Intravenous infusion of phospholipds into animals with experimentally-induced atheroma favourably influences this efflux (Adams et al., J. Path. Bact., 94:77-87, 1967), and apoAI/lecithin complexes are cleared from plasma at a rate similar to that of native HDL particles (Malmendier et al., Clin. Chem. Acta, 131:201-210, 1983).

The intravenous infusi n of apoAI in CHL fed rabbits reduces the areas of lesion involv ment by 50%.

This indicates that apoAI has a protective effect on atherosclerotic lesion formation, possibily by decreasing CHL uptake by the aortic wall (Maclejko and Mao, Artheriosclerosis, 2:407a, 1982; Mao et al., Fed. Proc. (USA), 42, no 7 pABSTRACT 357, 1983; Badimon et al., Cardiovascular Disease '86, 1986 ABSTRACT 81).

ApoAI is the major protein of HDL, about 70%, and is relatively abundant in plasma with a concentration of 1.0-1.2 mg/ml (Schonfield et al., J. Clin. Invest., 69:1072, 1982). Plasma apoAI is a single polypeptide chain of 243 amino acids whose primary sequence is known (Brew r et al., Biochem. Biophys. Res. Commun., 80:623-630, 1978). In its intracellular form apoAI is present as a 267 amino acid long precursor; the first 18 N-terminal residues of this longer protein are cleaved intracellularly by the signal peptidase of the rough endoplasmic reticulum. The newly excreted apoAI still contains a 6 amino acid long N-terminal extension; the conversion into its mature form is performed by a plasma and/or lymph specific protease (Zannis and Breslow, in Advances in Human Genetics, Harris and Hirschhorn eds., Plenum, 125-215, 1985).

The C-terminal part of the apoAI molecule has been shown to consist of repeat units of 11 or 22 amino acids (McLachlan, Nature, 267:465-466, 1979); the tandemly arrayed segments are not exact duplications, however, but amino acid subsitutions have generally conserved the chemical type of residues in corresponding positions of the repeats. These repeat segments are presumed to exist in amphipathic helical conformation (Segrest et al., FEBS Lett., 38:247-253, 1974), believed to be the major structural requirement for the main biological activities of apoAI, i.e. lipid binding and lecithin CHL acyltransferase (LCAT) activation. Two other functions of apoAI, acting as ligand for the recognition of HDL particles by a receptor, and removal of CHL from peripheral tissues, have not yet been related to specific s qu nces or

domains in the apoAI molecule.

The first described molecular variant of human apoAI is the Milano (apoAI-MI) mutant (Franceschini et al., J. Clin. Invest., 66:892-900, 1980). This is characterized by the Arg173-Cys substitution (Weisgraber et al., (J. Biol. Chem., 258:2508-2513, 1983), and was identified in 33 subjects belonging to the same kindred. All of them were heterozygous for the mutant apoprotein, which is transmitted as an autosomal dominant trait. They showed markedly reduced HDL-CHL concentrations and hypertriglyceridemia of a variable degree, negatively correlated with the HDL levels. No association between the mutant and specific pathological conditions could be established; indeed the affected subjects were apparently protected from the development of early atherosclerosis (Gualandri et al., Am. J. Hum. Gen., 1986, in press).

The amino acid substitution in the apoAI-MI modifies the structure of the mutant apoprotein, leading to a reduction of ordered alpha-helical structure and to an increased exposure of hydrophobic residues (Franceschini et al., J. Biol. Chem., 260:16321-16325, 1985). The remodelling of the mutant apoprotein significantly alters the lipid binding properties of the molecule which associates more readily than normal apoAI with lipids. Apoprotein/lipid complexes are similar to those formed by normal apoAI but are more easily destroyed by denaturing agents. The presence of a cysteine residue in the variant form allows the formation of complexes with apoAII, as well as apoAI dimers; these protein complexes are probably responsible for the formation of he anomalous HDL particles observed in the "affected" subjects. All these features of apoAI-MI may contribute to its accelerated catabolism, as well as to an efficient uptake capacity for tissue lipids.

Several other genetic variants of apoAI have been described so far (Breslow, Ann. Rev. Biochem., 54:699-727, 1985), but none of these is associated with any

pathological conditions or, in general, to significant alterations of lipoprotein metabolism in the carriers (Table 1 below).

ApoAI cDNA clones have been obtained by several laboratories (Breslow, Ann. Rev. Biochem., 54:699-727, 1985; Sharpe et al., Nucl. Acids Res., 12:3917-3932, 1984). The mRNA is about 890 base pairs (bp) long and presents a 5' untranslated sequence of 35bp, a coding sequence of 801bp (267 amino acids), a translation termination codon (TGA) and a 3' untranslated sequence of 54bp followed by a polyA tail. The complete cDNA nucleotide sequence is shown in Figure 1 of the accompanying drawings.

We have now found that recombinant DNA technology can be used successfully to produce proteins comprising apoAI or a genetic variant thereof. The variant maybe apoAI in which the 6-Ser has been replaced by Thr (apoAI-T6), apoAI-MI or a variant which combines the mutations of both apoAI-T6 and apoAI-MI (apoAI-T6/MI). The proteins can be detected by enzyme-linked immunoabsorbent assay (ELISA) with anti-apoAI anti-serum. The proteins which are produced may be in the form of fusion proteins in which the apoAI or genetic variant thereof is fused, at its N-terminus, to a carrier peptide.

Accordingly, the present invention provides an expression vector capable of expressing, in a transformed host, a protein which is capable of being detected by ELISA with anti-human apoAI antiserum and which has the formula (1):

Met-X-Y (1)

wherein X is a bond, a carrier peptide sequence which comprises a sequence derived from the N-terminal amino acid residues of beta-galactosidase or a sequence comprising one or more IgG-binding domains of Protein A, or the pro sequence of human apoAI; and Y represents the sequence of human apoAI or a genetic variant thereof.

The Met residue is attributable to the

translational start codon. Hosts transformed with such an expression vector and in which a said protein is capable of being expressed can be employed to prepare the protein of formula (1). The transformed host is cultured and the protein of formula (1) thus obtained is recovered. The proteins of formula (1) also form part of the invention.

In preferred embodiments, the invention relates to A) the construction of recombinant plasmids bearing the genes for:

1-apoAI (see Figure 1)
2-apoAI-T6
3-apoAI-MI
4-apoAI-T6/MI
5-apoAI with the following N-terminal extension Thr-Met-Ile-Thr-Pro-Ser-Phe-Asp-Gly-Ser-Met- (apoAI-RP5),
6-apoAI with the following N-terminal extension Thr-Met-Ile-Thr-Asn-Ser-Arg-Gly-Ser-Met- (apoAI-IP1),
7-the protein which combines apoAI-T6 and apoA1-RP5 (apoAI-RP5/T6),
8-the protein which combines apoAI-T6 and apoAI-IP1 (apoAI-IPI/T6),
9-the protein which combines apoAI-MI and apoAI-RP5 (apoAI-RP5/MI),
10-the protein which combines apoAI-MI and apoAI-IP1 (apoAI-IP1/MI),
11-the protein which combines apoAI-T6/MI and apoAI-RP5 (apoAI-RP5/T6/MI),
12-the protein which combines apoAI-T6/MI and apoAI-IP1 (apoAI-IP1/T6/MI) and
13-apoAI with the following N-terminal extension Arg-His-Phe-Trp-Gln-Gln (proapoAI)
14-the protein which combines proapoAI and apoAI-MI (proapoAI-MI)
15-the proteins of 541 amino acid residues with a N-terminal extension provided by staphylococcal protein A and which are composed of, from their N-terminus:

(the first 11 amino acids of the CRO protein of phage lambda)-(248 amino acids of staphylococcal protein A (residues 23 to 270))-(Pro-Gly-Asp-Ser-Thr)-(the last 17 amino acids of beta-galactosidase)-(the 17 amino acids which contain a recognition sequence for the proteolytic enzyme enterokinase and which are Gly-Asp-Pro-Glu-Phe-Val-Asp-Asp-Asp-Asp-Lys-Ser-Ser-Arg-Gly-Ser-Met)-(apoAI, apoAI-T6, apoAI-MI or apoAI-T6/MI); and

B) the expression of these genes in Escherichia coli (E. coli) strains. By expression of these genes, the following proteins are obtained:

- an apolipoprotein selected from Met-apoAI, Met-apoAI-T6, Met-apoAI-MI and Met-apoAI-T6/MI;
- a fusion protein wherein the amino acid sequence Met-Thr-Met-Ile-Thr-Pro-Ser-Phe-Asp-Gly-Ser-Met- is fused to an apolipoprotein selected from apoAI, apoAI-T6, apoAI-MI and apoAI-T6/MI;
- a fusion protein wherein the amino acid sequence Met-Thr-Met-Ile-Thr-Asn-Ser-Arg-Gly-Ser-Met is fused to an apolipoprotein selected from apoAI, apoAI-T6, apoAI-MI and apoAI-T6/MI;
- a proapolipoprotein proapoAI or proapoAI-MI; and
- a fusion protein composed of (the first 11 amino acids of the CRO protein of phage lamda)-(248 amino acids of staphylococcal protein A (residues 23 to 270))-(Pro-Gly-Asp-Ser-Thr)-(the last 17 amino acids of beta-galactosidase)-(the 17 amino acids which contain a recognition sequence for the proteolytic enzyme enterokinase and which are Gly-Asp-Pro-Glu-Phe-Val-Asp-Asp-Asp-Asp-Lys-Ser-Ser-Arg-Gly-Ser-Met)-(apoAI, apoAI-T6, apoAI-MI or apoAI-T6/MI).

In the accompanying drawings:

Figure 1 shows the complete nucleotide sequence corresponding to the mature form of human apoAI. Th s quence is shown in the mRNA s nse in the 5′ to 3′ direction; the low r line shows the amino acid sequence.

Figure 2 shows the reconstruction of the 5′ end of the gene coding for mature human apoAI. A DNA fragment from plasmid pAI/A, starting at the first Sau 3AI restriction site, was ligated to a synthetic oligonucleotide adapter assembled by annealing and ligating the single-stranded precursors 1-4. The resulting 790 bp fragment, flanked by two Bam HI restriction sites, codes for the mature apoAI molecule.

Figure 3 relates to the nucleotide sequence of pLS66 and pML11-20. The sequences show the junction between the ATG start codon of pFCE4$^+$ and the beginning of the apoAI gene before (pLS66, A) and after the deletion (pML11-20, B).

Figure 4 relates to the nucleotide sequence of pIL8-6 and pIL8-I. The sequences show the presence of the apoAI-MI mutation (C to T) in pIL8-I (A) and of the apoAI-T6 mutation (G to C) in pIL8-6 (B).

Figure 5 shows immunoblot analysis of pML11-20. Aliquots of the eluted material out of the affinity column and of a standard human HDL fraction were boiled and electrophoresed in duplicate on a 12.5% SDS-PAGE. Blotting on nitrocellulose filter was performed as described in the text. Lanes: 1- standard HDL; 2- eluate from the affinity column; 3- M_r standards.

Figure 6 shows immunoblot analysis of pRP5 and pUC9. Aliquots of bacterial cultures, bearing the wild type pUC9 or the recombinant pRP5 plasmids, induced with IPTG, were pelletted and resuspended in the appropriate buffer for gel electrophoresis in parallel with a standard human HDL fraction; after boiling samples were eletrophoresed on 12.5% SDS-PAGE. Blotting on nitrocellulose filter was performed as described in the text. Lanes: 1- M_r standards; 2- standard HDL; 3- pUC9; 4- pRP5.

Figure 7 shows the construction of plasmid pLM8, in which the boxes indicate the following: PROT A, AMP,

LAC Z and APOAI: genes coding for protein A, -lactamase, -galactosidase and human mature apoAI; CRO: sequence coding for the first 11 amino acids of the lambda cro protein; T: protein A transcription termination sequence; F1: phage f1 packaging sequence; ORI: origin of replication; Ek.AD: proteolytic site sequence recognized by enterokinase. EcoRI and ClaI sites in plasmid pLM8 are not unique.

Figure 8 shows the reconstruction of the 5' proapoAI sequence.

Figure 9 shows the construction of vector pFC33 which is capable of expressing proapoAI.

Figure 10 shows a gel electrophoresis and immunoblot analysis of the proapoAI expressed by pFC33. Lanes 1: HDL standard, Lanes 2: Non-recombinant strain, Lanes 3: proapoAI and Lane 4: Molecular Weight Standard.

An expression vector according to the invention is a vector capable of expressing DNA sequences and, in particular, the sequences of structural genes which the vector contains. The DNA sequences to be expressed are correctly positioned in relation to the upstream sequences which control their expression. Any suitable promoter system may be employed taking into account the protein to be expressed, the nature of the host, etc. Thus, a promoter selected from the P_L, lac, tac and trp promoters may be employed. In the present instance, the 5'-regulatory sequences are heterologous with respect to the apoAI or genetic variant thereof to be expressed. The regulatory sequences may be those of the lac or trp operon. In other words, an expression vector of the invention is capable of expressing a heterologous protein wherein the heterologous protein is apoAI or a genetic variant thereof. Preferred genetic variants are apoAI-T6, apoAI-MI and apoAI-T6/MI.

The expression vectors of the present invention, containing a DNA sequence encoding a protein of formula (1), are typically plasmids. They may be single-stranded.

Each vector is replicable, containing origin of replication. Preferred expression vectors are derived from plasmid pFCE4+ in the case of expression of apoAI or a genetic variant thereof, pUC8 or pUC9 in the case of expression of a fusion protein where the carrier sequence comprises a sequence derived from N-terminal amino acid residues of beta-galactosidase, pLM3 where the carrier sequence comprises one or more IgG binding domains of Protein A and pDS20 where proapoAI or a genetic variant thereof is being expressed.

In order to express the protein comprising apoAI or a genetic variant thereof, a suitable host is transformed with an expression vector according to the invention. A procaryotic or eurcaryotic host may be employed such as bacteria, yeast or a mammalian cell line. Typically, the host is a strain of E. Coli. A host should be chosen in which the desired protein is not degraded by cellular proteases. In general terms, therefore, a protein comprising apoAI or a genetic variant thereof may be obtained according to the following procedure:

1. Cloned apoAI cDNA is selected.
2. The cDNA is tailored so that the apoAI gene is provided with restriction sites at both ends so it becomes readily movable. This may entail removing the sequences coding for the signal peptide and propeptide.
3. The apoAI gene is inserted in an expression vector in the correct reading frame. Mutations T6 and/or MI may be introduced. The apoAI gene or variant gene may be the sole structural gene in an operon. Alternatively, it may be provided attached at its 5'-end to a DNA sequence encoding a carrier peptide where it is desired to express the apoAI or genetic variant thereof as a fusion protein.
4. A host in which the apoAI or genetic variant thereof is capable of being expressed is transformed with the expression vector.
5. The transformed host is cultured and the desired protein thus obtained is recovered.

Where a gene encoding apoAI or a genetic variant thereof is available without one or more introns, this gene may be inserted in frame in a vector under the transcriptional control of a promoter provided in the vector. The gene may be inserted immediately after a DNA sequence encoding a carrier peptide sequence. A DNA sequence encoding a protein of formula (I) can therefore be provided between translational start and stop codons in a vector providing the suitable promoter. Similarly, a gene encoding proapoAI or a genetic variant thereof can be inserted in a vector. Culture of a host transformed with the resultant expression vector leads to production of the desired protein.

The carrier peptide portion of a fusion protein may be derived from N-terminal amino acid residues of beta-galactosidase. It may be derived from, for example, up to the first fifteen N-terminal amino acid residues of beta-galactosidase. Preferably the residues are residues 5 to 15, more preferably residues 8 to 12. Preferred carrier peptides obtained in this way comprise the sequences:
Thr-Met-Ile-Thr-Pro-Ser-Phe-Asp-Gly-Ser-Met or
Thr-Met-Ile-Thr-Asn-Ser-Arg-Gly-Ser-Met

When fusion proteins incorporating such carrier peptides are expressed, they are preceded by Met. This applies too where the alternative carrier peptide comprising one or more of the IgG-binding domains of Protein A is employed. Such a carrier sequence may comprise the entire sequence of Staphylococcal protein A, or residues 23 to 270 of this protein. An amino acid sequence including a recognition site for a proteolytic enzyme such as enterokinase, factor X or collagenase, may immediately precede the sequence for apoAI or a variant thereof. A suitable sequence is:
Gly-Asp-Pro-Glu-Phe-Val-Asp-Asp-Asp-Asp-Lys-Ser-Ser-Arg-
-Gly-Ser-Met.

This is a recognition sequence for enterokinase. Other amino acids may be present in the carrier sequence.

For example there may be a linking sequence of up to 30 residues between the Protein A sequence and the sequence of a proteolytic enzyme recognition site. The N-terminal portion of the carrier sequence may comprise the first residues, for example up to 20 residues, of the structural gene naturally controlled by the promoter provided in the expression vector. Fusion proteins comprising a IgG-binding domain of Protein A are water-soluble, reasonably stable in an aqueous environment and are easily purified to homogeneity by affinity chromatography, e.g. on IgG-coupled Sepharose.

With or without the N-terminal extension provided by a carrier peptide, recombinant proteins of formula (I) are typically provided essentially free of other proteins of human origin. In other words, a protein is provided in essentially pure form, unaccompanied by protein with which it is ordinarily associated.

The protein comprising apoAI or a genetic variant thereof may be employed in a method of treatment of the human or animal body by therapy. More particularly, it may be used to lower plasma cholesterol and/or triglyceride levels. Thus, a protein may be used to combat atherosclerosis and cardio-vascular diseases such as coronary heart disease. The protein may be administered preventatively or for the amelioration/cure of an existing condition.

A protein produced according to the invention may therefore be provided as a pharmaceutical composition also comprising a pharmaceutically active carrier or diluent. Such compositions may be formulated in known manner. The protein may be adminstered parenterally, for example intravenously.

The following Examples illustrate the invention.

Example 1

MATERIALS AND METHODS

Strains and Media

Strains: E. Coli K12 JM101 (Messing et al., Nature, 314:309-321, 1981), MC 1061 (Casadaban and Cohen, J. Mol. Biol. 138:179-207, 1980), 71/18cI857 (Lorenzetti t al., Gene, 39:85-87, 1985), RL841 (Kenkel,Proc.Natl. Acad.Sci.USA, 82:488-492, 1985), CAG629 (rpoH165, lon-; C.A. Gross, Madison, Wisconsin).

Media: All strains were grown in LB medium with 50 ug/ml ampicillin when needed (LA medium). 1 mM IPTG was added to media to derepress the lac promoter when appropriate.

Enzymes, enzymatic reactions and DNA purification

Lysozyme was from Sigma (St. Louis, MO, USA). Restriction endonucleases were from Boehringer (Mannheim, DE), BRL Inc. (Gaithesburg, MD, USA) or New England Biolabs (Beverly, MA, USA); T4 ligase and all other enzymes were from Boehringer. All enzymatic reactions were performed according to the supplier's instructions unless otherwise specified. DNA fragments obtained by restriction endonuclease digestion were separated by electrophoresis in agarose horizontal gels or polyacrylamide vertical gels and extracted from the gel matrix by a modification of the electroelution method, using an ISCO Mod. 1750 concentrator (ISCO Co., Lincoln, NE, USA) and further purification on Elutip-d columns (Schleicher and Schuell, Keene, NH, USA).

Enzyme immunoassay for apoAI recognition in bacterial extracts (ELISA)

Pelleted cells were resuspended in lysis buffer (50 mM Tris-Cl pH 7.0, 30 mM NaCl, 0.1% NaN_3, 10 mg/l PhenylMethyl Sulfonyl Fluoride). The solution was made 1 mg/ml with lysozyme and was stirred at 4°C for 30 min. After 5 freeze-thawing cycles, the extracts were subjected to enzyme immunoassay as follows.

A standard 96 well microtit r plate was coated

with appropriate dilutions of anti-human apoAI antiserum raised in sheep (Boehringer, Mannheim, DE) overnight at 4°C. After 3 washes with 10mM Tris-Cl pH 8.0 containing 0.05% Tween-20 and 0.01% Merthiolate, 50 µl of serial dilutions of apoAI standard (TAGO Corp., Burlingame, CA, USA) or of bacterial extract were added and incubated for 1 h at 37°C. After 3 washes with the same buffer, 50 µl were added of an appropriate dilution of rabbit anti-human apoAI antiserum (Immuno Ltd., Dunton Green, Nr. Sevenoaks, Kent, GB) further purified by ammonium sulfate precipitation. After 1 hr at 37°C and 3 more washes, the wells were stained with a protein A-horseradish peroxidase kit supplied by New England Nuclear, (Boston, MA, USA) according to the instructions of the manufacturer.

Coupling of sheep anti-human apoAI to Affigel 10

Preparation of the sheep and anti-apoAI antibody (Boehringer, Manneheim, DE): 20 ml of undiluted antibody solution were dialyzed against 0.1 M Phosphate buffer pH 7, overnight at 4°C stirring continuously; then all debris were spun down saving the supernatant.

Preparation of Affigel 10 (Bio-Rad, Richmond, CA): 40 ml were equilibrated at room temperature before repeated (4-5 times) cycles of washing with half volume of isopropyl alcohol and then with double distilled water; the gel was dried down by filtration and transferred to a 50 ml falcon tube adding the dialized antibody. The tube was then incubated at 4°C for 8 hours stirring continuously and 1 ml of 1 M glycine ethyl ester was added; incubation continued overnight. The gel was then extensively washed with the following solutions:

- 100 ml of PBS + 0.1% sodium azide
- " " " 1 M acetic acid
- " " " 0.1 M phosphate buffer pH 7 + 0.5 M NaCl
- " " " PBS + 0.1% sodium azide;

packed into a column and equilibrated with the loading

buffer (PBS + 10mg/l PMSF + 10 mg/l Pepstatin A).

Immunoblot analysis

Samples were run on polyacrylamide slab gels (at the appropriate concentration) using the Laemmli method. Then the gels were transferred on nitrocellulose filters using a transblot apparatus (Bio-Rad) at 0.2 A for 4 hours at 4°C in 25 mM Tris Base, 192 mM Glycine, 20% Methanol. After transfer the filters were washed with distilled water and then incubated 1 hour with PBS + 3% BSA with gentle shaking. Filters were washed again with distilled water and incubated with the first antibody (the same used in ELISA as second antibody) diluted 1:250 with PBS, and incubated 1 hour at room temperature. After 2 washes with PBS and PBS + 0.05% Tween 20 the filters were incubated with the second antibody (anti rabbit IgG raised in goat conjugated with horseradish peroxidase; Bio-Rad) diluted 1:7500 for 1 hour at room temperature. After two more washes, filters were stained with 4-chloro-1-napthol.

RESULTS

5' end reconstruction of the apoAI gene

A full-length cDNA clone coding for apoAI (pAI/A) was obtained from a human liver cDNA library having the coding sequences for the signal peptide, the propeptide and the whole mature apoAI protein (Sharpe et al., Nucl. Acids Res., 12:3917-3932). We reconstructed the 5' end of the gene such that all sequences upstream of the first codon of mature apoAI were removed. For this purpose, plasmid pAI/A was digested with EcoRI and BamHI and an 890 bp fragment containing the cDNA insert was isolated and recovered from a 1% agarose gel. The fragment was restricted with Sau3AI under partial digestion conditions, and the mixture was ligated with a phosphorylated synthetic adapter which had been designed to reconstruct the coding sequence for the first 9 amino acids of the mature protein, optimizing the sequence according to codon usage in E. coli. The adapter was prepared as follows: four oligonucleotides were

synthesized by the phosphotriester method (Crea and Horn, Nucl. Acids Res., 8:2231-2348, 1980).

1. 5′ -GATCCATGGACGAGCC-3′
2. 5′ -ACCGCAGAGTCCATGG-3′
3. 5′ -CGGTGGCTCGTCCATG-3′
4. 5′ -GATCCCATGGACTCTG-3′

All four oligonucleotides were ligated together in equal molar amounts, the ligation mixture was treated with Sau3AI and the adapter was eluted and purified from a polyacrylamide gel. The resulting sequence of the adapter is illustrated in Fig. 2.

The ligation mixture containing the purified adapter and the partial Sau3AI-cut apoAI fragment was digested with BamHI. A 790 bp fragment was purified from a polyacrylamide gel and ligated to BamHI-cut pAT153-PvuII8 treated with Calf Intestine Phosphatase. After transformation of MC 1061, recombinants were screened by hybridization with phosphorylated oligonucleotide 3 as a probe. Positive colonies were isolated and further charaterised by restriction enzyme digestion and sequencing by partial chemical degradation according to Maxam and Gilbert (Methods Enzymol., 65:499-560, 1980). The plasmid having the adapter in the correct orientation was designated pAI/12.

Thus reconstructed, the 5′ end of the apoAI gene was preceeded by an ATG start codon and a BamHI cohesive end. Using this strategy, the mature apoAI coding sequence could be moved to any expression vector carrying a BamHI site after the Shine-Dalgarno sequences.

Construction of recombinant vectors using pFCE4+

The BamHI fragment containing the modified apoAI gene was purified and subcloned into the BamHI site of pFCE4+ (Lorenzetti et al., 1985). The resulting construction with the corr ct rientation of the insert was verified by the dideoxy chain termination sequencing (fig.3A). This plasmid was named pLS66.

Using this strategy, the apoAI gene was inserted out of frame with respect to the ATG start codon already present in the vector. Thus, the next step was to restore the reading frame by positioning the apoAI gene immediately after this ATG codon.

In-frame positioning

Using the pFCE4 system the in-frame positioning of a cloned gene can be performed using "bridge" oligonucleotides, having sequences complementary to the stretches of bases flanking the region to be deleted on either side (Sollazzo et al., Gene, 37: 199-206, 1985).

To do so, we synthesized, by the phosphotriester method, a single-stranded oligonucleotide having the following sequence:

5' -CTTACATATGGACGAGCC-3'.

This oligonucleotide should anneal with its 5' end to the region immediately preceding and including the ATG of the vector (nucleotides 1 to 10), and with its 3' end to the first 8 nucleotides of the apoAI gene, thus looping out the 8 extra nucleotides present in pLS66 (Fig. 2). The ssDNA of pLS66 was prepared as described (Kunkel, Proc. Natl. Acad. Sci. USA, 82: 488-492, 1985) using E. coli RL841 as host. This bacterial strain is a derivative of E. coli RZ1032 containing plasmid pCI857 (M. Zabeau) which produces a temperature sensitive repressor for the P_L promoter present in pFCE4.

Using this protocol, the ssDNA produced by this strain contained several uracil residues in place of thymine, thus acting as a normal functional template "in vitro" but not being biologically active upon transformation into a wild-type (ung+) E. coli 71/18cI857 strain, which is the normal host for these plasmids.

0.1 pmoles of pLS66 ssDNA were annealed with 2 pmoles of kinased mutagenic oligonucleotide (a 20 fold excess) at 56°C for 30 min in 15 mM Tris-Cl, pH 8.5, and 15 mM $MgCl_2$. Aft r cooling to room temperature the

elongation-ligation mixture was added and the reaction was continued at 15°C overnight. The elongation reaction mixture was as follows (final concentrations): 10 mM Tris-Cl, pH 8.5; 10mM $MgCl_2$; 0.5 mM ATP; 0.05 mM dNTPs; 5 mM DTT; 1 unit of DNA polymerase, Klenow fragment and 1.5 units of T4 DNA ligase.

After incubation the reaction mixture was phenol extracted, isopropanol and ethanol precipitated; 71/18cI1857 competent cells were transformed and plated on selective medium, LA. In order to identify clones that contained the desired deletion, 150 colonies were picked, grown on ordered plates and transferred onto nitrocellulose filters. The hybridization was performed at room temperature in 6x SSC and 10x Denhardt's using the mutagenic oligonucleotide as a probe. The filters were then washed in 6x SSC and 0.1% SDS at two different temperatures: the first time at room temperature, to check for hybridization efficiency, and then at 50°C, i.e. 4°C below the theoretical melting temperature. This was calculated according to the following formula, Tm = 4xGC + 2xAT, where GC and AT are the numbers of pairings formed by the oligonucleotide with the mutagenized template (Norrander et al., Gene, 26: 101-106, 1983). After each washing the filters were exposed with X-ray sensitive films. Candidate mutant colonies, yielding positive signals also at a stringent temperature, were confirmed by dideoxy chain-termination sequencing (Fig. 3B). The efficiency of this protocol is rather high, about 50%. pFCE4+ with the apoAI gene correctly positioned in frame was named pML11-20.

Construction of apoAI variants T6 and MI

Both pLS66 and pML11-20 were used as templates for the construction of the two variants apoAI-T6 and apoAI-MI. We decided to use both templates to obtain at the sam time, the variant genes correctly positioned in frame and in a transposable BamHI fragment.

For this purpose two single oligonucleotides were synthesized by the usual protocol:

1. 5′ -CACCGCAGACTCCATGG-3′ for the T6 mutation
2. 5′ -GCGCCAGTGCTTGGC-3′ " " MI "

Indicating with number 1 the first nucleotide of the first codon of mature apoAI, oligonucleotide 1 should anneal from residues 8 to 24, with a mismatch in position 17 (G to C). Oligonucleotide 2 shoud anneal from residu s 510 to 524, with a mismatch in position 517 (C to T).

The mutagenesis experiments were performed using the same protocol described for the in frame positioning (Fig. 4A and B). For the double mutants we first obtained the T6 mutation and then added the MI mutation.

The resulting plasmids were named:

pIL5-6 = apoAI-T6 between two Bam HI sites
pIL5-I = apoAI-MI " " " " "
pIL5-6I = apoAI-T6/MI between two Bam HI sites
pIL8-6 = apoAI-T6 in frame
pIL8-I = apoAI-MI " "
pIL8-6I = apoAI-T6/MI in frame

Construction of the variants apoAI-RP5 and IP1

The DNA fragments containing the apoAI gene and its variants T6 and MI were excised from plasmids pLS66, pIL5-6, pIL5-I and pIL5-6I by Bam HI digestion and were purified on a 1% agarose gel as described. The fragments were then ligated to pUC8 and pUC9 (Vieira and Messing, Gene, 19: 259-268, 1982), both linearized with Bam HI. After transformation of JM101 competent cells, recombinant plasmids were identified by the rapid disruption method and verified by cutting isolates with Bam HI. Two opposite orientations of the insert were distinguished by cutting with Hind III and Xho I.

Constructions with pUC8 had the apoAI gene already in frame, immediately preceeded by a methionine residue and the first 9 amin acids of the alpha-peptide from the bacterial beta-galactosidase gene; this resulted

in a 10 amino acid extension at the N-terminal of the apoAI molecule of beta-galatosidase.

All the recombinant pUC9 derivatives, containing the apoAI genes in the correct orientation, required more work to put the apoAI gene in frame. They were first cut with the restriction enzymes Hind III and Sal I between the ATG translation start and the apoAI gene. The cohesive ends generated by cutting were filled in with DNA polymerase (Klenow fragment) and then religated with T4 DNA ligase. This resulted in the deletion of 10 nucleotides at the multiple cloning site of pUC9 , in front of the apoAI gene, thus restoring the correct reading frame.

The recombinant plasmids were named:

a) pUC8 series

pIPI = with apoAI-IPI
pIPI-6 = " " " /T6
pIPI-I = " " " /MI
pIPI-6I = " " " /T6/MI

b) pUC9 series

pRP5 = with apoAI-RP5
pRP5-6 = " " " /T6
pRP5-I = " " " /MI
pRP5-6I = " " " /T6/MI

The rationale to obtain the extended variants was that expression levels were increased by the presence of the N-terminal end of bacterial beta-galactosidase, the sequence naturally occurring after the regulatory elements present in the plasmids.

Expression in E. coli strains

1) All recombinant pFCE4 derivatives were transformed in the bacterial host CAG629 bearing the plasmid pCI856 (already described in the "In Frame Positioning" section).

Overnight cultures grown at 32°C were diluted 1:10 in LA and grown in shaker flasks till OD_{650} 0.5-0.6, then the temperature was shifted at 42°C and incubation was

continued for 1-2 hours. After induction, cells were chilled on ice for 10 min. and pelleted by centrifugation. The pellets were then treated as described in the "ELISA" section to break the cells. The cell extracts were centrifuged at 16.000 rpm for 30 min. at 4°C and the supernatant applied to the affinity column described in "Materials and Methods". After extensive washing the retained proteins were eluted with 1M acetic acid. The eluted fractions were assayed by immunoblot analysis (Fig. 5) and by "ELISA"; preliminary results obtained with pML11-20 averaged around 0.2 mg of apoAI per liter of culture.

Similar results were obtained with all other constructions bearing mutations apoAI-T6, apoAI-MI and apoAI-T6/MI.

2) All recombinant pUC8/9 derivatives were transformed in the bacterial host MC1061.

Overnight cultures grown at 37°C were diluted 1:100 in LA and grown in shaker flasks for 1 hour prior the addition of 1 mM IPTG, then incubation was continued for an other 4-6 hours. After this time the cells were chilled on ice for 10 min. and pelleted by centrifugation. From this step on, the extraction procedures were the same as those described for the pFCE4 derivatives. Crude extracts of cells containing pIPI and pRP5 were assayed by immunoblot analysis (Fig. 6) and by "ELISA"; preliminary results averaged around 10 mg of apoAI per liter of culture.

Similar results were obtained with all other constructions bearing mutations apoAI-T6, apoAI-MI and apoAI-T6/MI in combination with both apoAI-RP5 and apoAI-IP1.

Example 2

Other variants were constructed using the vector pRIT2T (Pharmacia, Sweden) in which a 949 bp fragment bearing the apoAI gene, was inserted into the Sma 1 site of that v ctor. The protein expressed by this plasmid was

composed of 541 amino acids. These are, from the N-terminal end, the first 11 amino acids of the CRO protein of phage lambda, followed by 248 amino acids of the sequence for staphylococcal protein A (from residues 23 to 270), 5 unusual amino acids (Pro-Gly-Asp-Ser-Thr) due to the junction with the last 17 amino acids of the beta-galactosidase of E. coli and 17 amino acids, which contain the recognition sequence for the proteolytic enzyme enterokinase, Gly-Asp-Pro-Glu-Phe-Val-Asp-Asp-Asp-Asp-Lys-Ser-Ser-Arg-Gly-Ser-Met), which immediately precede the sequence of the mature human apoAI (named CPA-AI) or its variants apoAI-T6, apoAI-MI, apoAI-T6/MI. These chimeric proteins are stable and soluble in aqueous solutions.

The interaction of CPA-AI with rat hepatocytes and with mouse macrophages in culture has been studied. Incubation of ^{125}I-labelled CPA-AI with the cultured cells at 4°C shows that the hybrid protein binds to the cell surface with binding parameters similar to HDL. The hybrid protein is taken up by the cells at 37°C and is shown to be internalized. This behaviour resembles that of natural apoAI when associated with lipids to form HDL particles.

MATERIALS AND METHODS

Materials

HDL ($1.09<d<1.21$ g/ml) was prepared from human plasma on a discontinuous KBr gradient. The HDL was extensively dialysed against PBS, 1mM EDTA to remove the KBr and stored at 4°C. Human transferrin (Sigma) was loaded with iron according to the method of Klausner et al (J. Biol. Chem. 258, 4715-4724, 1983). BSA was essentially IgG free (Sigma, A 7638). Staphylococcus aureus protein A was from Pharmacia. Hanks' balanced salt solution containing phenol red was from Gibco.

Cells and cell culture

Fao cells, a rat hepatoma cell line established and characterized by Deschatrette and Weiss (Biochemie 56, 1603-1611, 1974), were cultivated in monolayer in Coon's

modified Ham's F12 medium (Seromed) supplemented with 5% fetal calf serum (FCS), 100 units/ml penicillin, 100µg/ml streptomycin, 2mM glutamine and 10mM HEPES buffer pH 7.4. J774 cells, a mouse macrophage cell line (Ralph et al, J. Immunology 114, 898-905, 1975), were maintained in spinner suspension culture in D-MEM medium containing 4.5% FCS, penicillin, streptomycin, glutamine and HEPES buffer pH 7.4 at concentrations as below.

Bacterial strains and plasmids

The bacterial hosts were E. coli strains HB101 (Boyer and Roulland-Dussoix, J. Mol. Biol. 41, 459-472, 1969) and RR1 M15 (Langley et al. Proc.Natl.Acad.Sci.USA 72,1254-1257, 1975). The plasmids used were pNF2690 (Nilsson et al, EMBO J. 4, 1075-1080, 1985) and pLM3 (Monaco et al Atti Convegno congiunto A.G.I.-S.I.B.B.M., 195-196, 1985); pRIT2T is sold by Pharmacia, Sweden.

DNA constructions

Restriction enzymes, Klenow DNA polymerase and T4 DNA ligase were purchased from Boehringer Mannheim and us d according to the supplier's specifications. Transformation of E. coli was performed according to Morrison (Methods Enzymol. 68, 326-331, 1979).

Expression of the hybrid gene

The hybrid gene, under the control of the lambda P_R promoter, was expressed essentially in accordance with Zabeau and Stanley (EMBO J. 1,1217-1224, 1982). Bacteria were grown at 30°C to OD_{600} = 0.9. The temperature was quickly raised to 42°C by addition of an equal volume of broth pre-heated at 54°C. Cultures were incubated at 42°C for 90 min before harvest. Total cell lysates were loaded on gels as described by Zabeau and Stanley (1982).

Purification of the hybrid protein

The method of Marston et al (Biotechnology 2, 800-804, 1984) was followed f r the purification of the hybrid protein. Bact rial pellets were resusp nded in 3 v lumes (w/v) of buffer A (50mM Tris/HCI pH 8.0, 50 mM

NaCl, 1mM EDTA) and sonicated 5 X 20 sec on ice. After centrifugation (10,000 rpm, 10 min at 4°C in a Sorvall SS34 rotor), the pellet was resuspended in 9 volumes (w/v) of buffer A containing 0.5% Triton X-100,10 mM EDTA and left at room temperature for 5 min. The suspension was centrifuged as above and the pellet was resuspended in 9 volumes (w/v) of buffer A containing 8 M urea and incubated at room temperature for 1 h.

The urea suspension was added to 9 volumes (v/v) of 50 mM potassium phosphate buffer, pH 10.7, 50 mM NaCl, 1 mM EDTA and stirred for 30 min at room temperature while keeping the pH at 10.7 by addition of KOH. The suspension was then neutralised and dialysed at 4°C against buffer A. The dialysed suspension was centrifuged as above; the hybrid protein in the clear supernatant was already reasonably pure, the average yield of hybrid protein at this stage was 50-60 mg per liter of bacterial culture. A further purification was achieved by affinity chromatography on IgG-Sepharose 6 FF (Pharmacia). The sample was loaded onto the column, washed sequentially with PBS containing 0.05% Tween 20, PBS and 5mM $AcONH_4$, pH 5 and eluted with 1M $AcOH/AcONH_4$, pH 2.8. The purified protein was dialysed against buffer A.

Calibration using a Biogel 1.5 A gel filtration column

A Biogel 1.5 A column from Bio Rad (40cm x 1cm) was equilibrated in 50mM Tris/HCl pH 8.0, 50mM NaCl, 1mM EDTA. The sample was eluted at a flow rate of 3 ml/h and 250µl fractions were collected. The column was calibrated with dextran blue (2,000 kD), thyroglobulin (699 kD), ferritin (440 kD), HDL (285 kD) and aldolase (158 kD). The Mr of the hybrid protein was determined using 1.25µg of [^{125}I]-CPA-AI or 800µg of unlabelled CPA-AI.

Iodination of proteins

Proteins were iodinated using Iodo-Gen reagent (Pierce Chemical Co.). Th iodination reaction contained

100µg Iodo-Gen, 1mg protein in 200µl PBS and 1mCi carrier free Na [^{125}I], 16.5mCi/µg (Amersham International). After 10 min at 4°C-free iodine was separated from the protein on a 10 ml Sephadex G50 column. The column was eluted with PBS (0.2% BSA) for HDL, transferrin and IgG and 50mM Tris/HCl pH 8.0, 0.15M NaCl, 1mM EDTA, 0.2% BSA for the hybrid protein. The hybrid protein, IgG and transferrin were used without dialysis. It was necessary to extensively dialyse HDL against PBS, 0.5mM EDTA until less than 5% of the label was soluble in 10% (w/v) trichloroacetic acid. Less than 5% of the label was associated with lipid as determined after lipid extraction with chloroform/methanol. The specific activity obtained was 200-400 cpm/ng protein.

Building assays

Binding assays on suspension cells were performed for 2h at 4°C. To obtain a suspension of Fao cells, the monolayers were incubated with PBS, 1mM EDTA for 30 min at 37°C. These cells or the suspension grown J774 macrophages were then washed 3 times by pelleting at 1000g x 5 min at 4°C and resuspending in 3ml Hanks/BSA. Each binding assay contained 1 x 10^6 cells in 1ml Hanks/BSA and the indicated amount of [^{125}I]-apoA-1-PA or [^{125}I]-HDL. After 2h at 4°C, the cells were washed 4 times in Hanks/BSA, transferred to Eppendorf tubes and radioactivity counted in a gamma counter. For competition experiments 10µg iodinated ligand were competed by 10-800µg of either unlabelled HDL, CPA-AI, protein A or transferrin, each of which were added to the cells at 4°C, 30 min prior to the addition of iodinated ligands.

Ligand blot of HDL and CPA-AI

J774 cells (2 x 10^8) were washed 2x in PBS and homogenized in 10mM Tris/HCl, pH 7.4, 0.1mM EDTA. A post nuclear supernatant (PNS) was prepared by centrifugation at 2000g for 10 min. The PNS was extracted with 1% (v/v) Triton X-114 (Bordier, J. Bi l. Chem. 256, 1604-1607,

1981). The detergent phase was mixed with an equal volume of sample buffer containing dithiothreitol, boiled and then alkylated with iodoacetamide. Approximately 1mg protein per lane was applied to a 5-15% SDS polyacrylamide gel. The gel was blotted onto nitrocellulose and the bands visualized by Ponceau S staining. The nitrocellulose strips were quenched for 6 h in Hanks containing 10% (w/v) low fat milk powder. Ligands, either [^{125}I]-CPA-AI or [^{125}I]-HDL at 12.5μg/ml were bound in Hanks/milk for 16h. The strips were then washed 4x with Hanks/milk and the ligands detected with either rabbit antiserum against: human HDL, CPA-AI or protein A (at 1/50 serum dilution) washed, and incubated in the presence of anti rabbit IgG peroxidase which was revealed with the diaminobenzidine reaction (Burnette, Anal. Biochem. 112, 195-203, 1981). The dried blot was then exposed to X-ray film.

Analytical methods

Amino-terminal sequencing was by automated Edman degradation performed in a gas-liquid phase microsequencer constructed as described by Frank and Trosin (H. Tschesce (editor): Modern Methods in Protein Chemistry, Walter de Gruyter & Co., Berlin, 287-302, 1985) according to Lottspeich (Hoppe Seyler's Z. Physiol.Chem. 361, 1829-1834, 1980). To determine the carboxy-terminal sequence of the hybrid protein, carboxypeptidase ε, A and/or B were used according to Tadros et al (Eur. J. Biochem. 138, 209-212, 1983). For amino acid analysis the protein was hydrolyzed according to Tadros et al (Eur. J. Biochem. 127, 315-318 1982) and the amino acid composition determined using an automated amino acid analyzer (Durrum D-500). Tryptophan and cysteine were not determined.

For microscopy, samples of HDL and CPA-AI were negatively stained using 2% phosphotungstic acid. Protein concentration was assayed by the method of Bradford (Anal. Biochem. 72, 248-254, 1976).

RESULTS

Construction of plasmid pLM8 carrying the gene encoding the hybrid protein CPA-AI

The vector pRIT2T is a derivative of pRIT2 (Nilsson et al, 1985), designed for temperature-inducible expression of intracellular hybrid proteins in E. coli. The construct contains the IgG-binding domains of Staphylococcal protein A which are under the control of the lambda P_R promoter. A multiple cloning site facilitates the insertion of foreign genes at the 3′ end of protein A. The protein A transcription termination sequence is inserted immediately downstream from the multiple cloning site. Plasmid pLM3 contains the E. coli lacZ gene fused in frame to the mature gene for human apoAI (Monaco et al 1985).

The plasmid pLM8, carrying the fusion between PA and apoAI, was constructed as described in Figure 7. The 949 bp fragment flanked by the restriction sites EcoRI and ClaI was isolated from pLM3 and the protuding ends were filled in by Klenow DNA polymerase. The fragment was ligated to the SmaI linearized pRIT2T. The resulting plasmid pLM8 carries an open reading frame, coding for a fusion protein of 541 amino acids, composed of the gene for protein A at the 5′ end and the mature human apoAI gene at the 3′ end separated by a specific sequence for proteolytic cleavage.

Expression, isolation and purification of the hybrid protein

E. coli cells carrying pLM8 in the presence of pNF2690, a compatible plasmid coding for the temperature sensitive C1 857 lambda repressor mutant, were capable of expressing large amounts of hybrid protein. When grown at 42°C, the amount of the induced CPA-AI represents about 20% of the total. The apparent Mr of the hybrid protein is, as xpected, 62 kD on SDS-PAGE. Standard methods for extraction (sonication, fre ze-thawing, lysozyme-Triton

X-100) provided unsuccessful. A denaturation procedure, involving incubation of the sonicated bacteria in 8M urea followed by addition of an alkaline buffer, has been adopted. After renaturation by neutralization and dialysis, the suspension was centrifuged and the clear supernatant contained the fusion protein at greater than 90% purity. Further purification was achieved by affinity chromatography by IgG Sepharose.

Characterization of the hybrid protein

The identity of the hybrid protein was confirmed by recognition by a specific apoAI antibody prepared in sheep. Protein A does not recognize the sheep antibodies.

The amino-terminal sequence of the first eight amino acids and the carboxy-terminal sequence of the last three amino acids were in agreement with the known sequence of apoAI and protein A. Also the amino acid composition analysis was in agreement with the expected composition.

To quantitate the binding of the hybrid protein to the cell surface (see below) it was necessary to determine its native Mr. This was performed by gel filtration using Biogel 1.5 A. The peak fraction from the column elution gave a Mr of 316 kD, which corresponds to 5 times the momomer Mr of 62 kD. The broad profile of the hybrd protein elution indicated that other multimers were also present, as well as a small amount of aggregated material in the void volume. The average size of 316 kD was used as a native Mr for all experiments. This is compared to 285 kD for HDL used as standard on the same column. The elution profile of the iodinated hybrid protein was similar to that obtained for unlabelled hybrid protein.

HDL and hybrid protein particles were examined after negative staining with the electron microscope. The micrographs showed a homogeneous population of particles with a diameters ranging from 9-12 nm. The hybrid protein particles have a slightly larger size than HDL, confirming

the results obtained by gel filtration.

Comparison of the biological activity of the hybrid protein and HDL

The hybrid protein purified from E. coli and HDL purified from human plasma were used in parallel to study their capacity to bind cell surface receptors. Several cell types were investigated; a mouse macrophage (J774) and a rat hepatocyte (Fao) cell lines. There is evidence that these two cell types process HDL differently. Cholesterol exchange occurs in macrophages and degradation in hepatocytes.

Binding data at 4°C

Iodinated ligands were allowed to bind to the cells at 4°C for 2 h. A plateau in the amount bound had been reached by 30 min. Competition with 500µg of unlabelled ligand was used to evaluate the amount which was non-specific and this value was subtracted from the total binding curve to give the value of specific binding. The specific binding values were used to calculate the affinity by Scatchard analysis (Scatchard, Ann. N.Y. Acad. Sci. 51, 660-672, 1949). With both HDL and the hybrid protein the binding was specific and of high affinity. The high affinity binding has a Kd of $2.8-3.0\times10^{-8}$M for HDL and $3.5-4.9\times10^{-8}$M for the hybrid protein with either the J774 or Fao cells. The results are shown in Table 2 below. In addition, there is a low affinity component not completely competed by unlabelled ligand with both the HDL and hybrid protein.

The high affinity binding component in both cell types gives a maximal binding of 90-155ng/1×10^{6} cells: this represents $1.9-3.6\times10^{5}$ sites per cell. The number of binding sites per cell and the binding affinities were remarkably similar for both HDL and the hybrid protein. This suggests that a single receptor is involved and that the apoAI component alon can account for the r ceptor mediated binding of HDL.

Competition studies

Additional evidence for the specific binding of apoAI comes from competition studies. Both HDL and the hybrid protein competed effectively with each other for binding to J774 and Fao cells. The competition was efficient until the non-specific component is reached. Protein A was unable to compete for hybrid protein or HDL binding. As an independent control, transferrin was used in competition studies. Although transferrin binds via the transferrin receptor to the cell surface, it could not compete with the binding of HDL or the hybrid protein.

Protein A and IgG binding activities

Since the hybrid protein also contains protein A, it was important to determine whether protein A itself could bind to the cells. Protein A was iodinated to a similar specific activity and assayed for binding to both J774 and Fao cells. Even at high concentrations of protein A (100µg/ml), specific binding was not detected. The amount of cell-associated radioactivity was less than the non-specific binding of the hybrid protein.

A precaution was necessary in carrying out these experiments with the hybrid protein because J774 cells express Fc receptors for IgG. Therefore [^{125}I]-rabbit IgG binds to the cell surface Fc receptors with high affinity (Kd = 1×10^{-8}M). When [^{125}I] CPA-AI was bound in the presence of rabbit IgG, the amount of hybrid protein bound increased by 2.7 fold. Conversely, when [^{125}I]-rabbit IgG was bound in the presence of hybrid protein, the binding was also increased (1.6 fold). Apparently, the complex between IgG and CPA-AI can bind to either the Fc receptor or to the HDL receptor. To study only the HDL receptor, all the binding experiments were carried out in IgG free media. The cells were washed 3 times in Hanks buffer suppl mented with BSA (essentially IgG free) to remove the IgG present in the culture medium.

Charact rization of the HDL receptor

J774 cells were treated with trypsin at 4°C prior

to assaying for binding of HDL, the hybrid protein or transferrin. The trypsin treatment did not decrease the HDL or hybrid protein binding. However the binding of [^{125}I]-transferrin was reduced to 38% of the control. The HDL receptor is relatively trypsin resistant compared with the transferrin receptor.

To identify the receptor involved in the binding of HDL and the hybrid protein a ligand blot was perform d. A Triton X-114 extract of a PNS from J774 cells was subjected to SDS-PAGE under reducing conditions and transferred to nitrocellulose. [^{125}I]-HDL and [^{125}I]-CPA-AI each bound to a band of about 110 kD. This band was identified either directly by visualisation of the [^{125}I]-labelled ligands on an autoradiograph of the filter or indirectly by antibody detection using a rabbit antiserum against HDL and visualisation with sheep anti rabbit IgG peroxidase. For HDL, there was a very high background f iodinated label on the nitrocellulose which obscured th detection of a specific band on the autoradiograph.

EXAMPLE 3

Human apoAI is synthesized as a precursor protein, preproapo AI of 267 amino acids. The 18 amino acids long prepeptide is cleaved during secretion, leaving a proprotein denoted proapoAI. ProapoAI consists therefore of a six aminoacids N-terminal extension, Arg-His-Phe-Trp-Gln-Gln, followed by the mature protein.

In order to express proapoAI, the 5′ of the apoAI gene was synthetically reconstructed as shown in Figure 8. A Nde I-Bam HI oligonucleotide coding for the first 15 amino acids of proapo AI including the 6 amino acids propeptide was synthesized. The synthetic DNA included an Nco I site immediately before the Bam HI site. An Eco RI-Sal I fragment carrying the trp promoter from plasmid pDR720 (Pharmacia, Sweden; Russel and B nn t, G ne 20,231,1982) was ligat d to a synthetic Sal I-Nde I fragment coding for the lambda cII Shine-Dalgarno sequence

to obtain the Eco RI-Nde I 107 bp fragment illustrated in the figure. This DNA piece was ligated to the fragment coding for the proapoAI 5′ sequence and subcloned into M13mp8 (Messing et al, Proc.Natl.Acad.Sci.USA 74,3642, 1977) to be sequenced.

In order to express the recombinant proapoAI, an expression vector was constructed as illustrated in Figure 9. An Eco RI-Nco I fragment harbouring the expression signals followed by the 5′ end of the proapoAI gene was cut from the recombinant MI3mp8 (See Fig. 8) and ligated to the two following fragments:

a) the large fragment of plasmid pDS20 (Duester et al, Cell 30, 855, 1982) cut with Eco RI-Bam HI

b) an Nco I-Bam HI fragment from pML11-20 containing the rest of the apoAI sequence.

The resulting plasmid pFC33 was able to express a proapoAI protein, or more specifically Met-proapoAI, under the trp promoter in an E. coli B strain (Delbruck, 1946; Bacterial viruses or bacteriophages Biol. Rev. 21:30-40). For induction, cells were grown overnight in a LB medium with ampicillin. The next day, cells were diluted into M9 medium without tryptophan and harvested after 6 hours at 37°C.

Figure 10 shows a gel electrophoresis and an immunoblot analysis of proapoAI. Aliquots of bacterial cultures were pelleted and resuspended with gel electrophoresis buffer in parallel with a standard human fraction. After boiling, samples were electrophoresed on 12.5% SDS-PAGE. Blotting on nitrocellulose filter was performed as described in Example 1.

TABLE 1

ZYGOSIS	NAME	MODIFICATION	BIOCHEMICAL ALTERATIONS
HETEROZYGOTE	MILANO	Arg 173 --> Cys	FORMS DIMERS AND COMPLEXES WITH Apo AII
"	GIESSEN	Pro 143 --> Arg	60-70% OF NORMAL LCAT ACTIVATION ABILITY
"	MARBURG (MUNSTER 2)	Lys 107 --> O	40-60% OF NORMAL LCAT ACTIVATION ABILITY
	MUNSTER 3 :		
"	FAMILY A	Asp 103 --> Asn	NONE
"	B	Pro 4 --> Arg	
"	C	Pro 3 --> His	

ApoAI genetic variants naturally occurring in the human population

Table 2: Binding of [^{125}I]-HDL and [^{125}I]-CPA-AI to either Fao or J774 cells

	Mr	Binding Kd		High affinity
		µg/ml	M	Sites/cell
Fao				
HDL	285,000	8.6	$3.0x10^{-8}$	362,000
CPA-AI	316,000	11.0	$3.5x10^{-8}$	248,000
J774				
HDL	285,000	8.0	$2.8x10^{-8}$	190,000
CPA-AI	316,000	15.5	$4.9x10^{-8}$	277,000

CLAIMS

1. An expression vector capable of expressing, in a transformed host, a protein which is capable of being detected by ELISA with anti-human apoAI antiserum and which has the formula (1)

Met-X-Y (1)

wherein X is a bond, a carrier peptide sequence which comprises a sequence derived from the N-terminal amino acid residues of beta-galactosidase or a sequence comprising one or more IgG-binding domains of Protein A, or the pro sequence of human apoAI; and Y represents the sequence of human apoAI or a genetic variant thereof.

2. An expression vector according to claim 1, wherein the said protein is Met-apoAI, Met-apoAI-T6, Met-apoAI-MI or Met-apoAI-T6/MI.

3. An expression vector according to claim 2, which is derived from plasmid pFCE4+.

4. An expression vector according to claim 3, which is plasmid pML11-20, pIL8-6, pIL8-I or pIL8-6I.

5. An expression vector according to claim 1, wherein the carrier peptide sequence comprises a sequence derived from up to the first fifteen N-terminal amino acid residues of beta-galactosidase.

6. An expression vector according to claim 5, wherein the carrier peptide comprises the sequence Thr-Met-Ile-Thr-Pro-Ser-Phe-Asp-Gly-Ser-Met or Thr-Met-Ile-Thr-Asn-Ser-Arg-Gly-Ser-Met.

7. An expression vector according to claim 6, which is derived from plasmid pUC8 or pUC9.

8. An expression vector according to claim 7, which is plasmid pIPI, pIPI-6, pIPI-I, pIPI-6I, pRP5, pRP5-6, pRP5-I or pRP5-6I.

9. An expression vector according to claim 1, wherein the carrier peptide comprises staphylococcal protein A r residues 23 to 270 there f.

10. An expression vector according to claim 1,

wherein the carrier sequence comprising one or more IgG-binding domains of Protein A is fused to the sequence of human apoAI or a genetic variant thereof via a recognition sequence for a proteolytic enzyme.

11. An expression vector according to claim 10, wherein the carrier peptide sequence is composed of (the first 11 amino acids of the CRO protein of phage lamda)-(248 amino acids of staphylococcal protein A (residues 23 to 270))-(Pro-Gly-Asp-Ser-Thr)-(the last 17 amino acids of beta-galactosidase)-(the 17 amino acids which contain a recognition sequence for the proteolytic enzyme enterokinase and which are Gly-Asp-Pro-Glu-Phe-Val-Asp-Asp-Asp-Asp-Lys-Ser-Ser-Arg-Gly-Ser-Met).

12. An expression vector according to claim 1, wherein the carrier peptide sequence comprises one or more IgG-binding domains of Protein A and which is derived from plasmid pLM3.

13. An expression vector according to claim 12, which is plasmid pLM8.

14. An expression vector according to claim 1, wherein the pro sequence of human apoAI is fused to the sequence of human apoAI or a genetic variant thereof and which is derived from plasmid pDS20.

15. An expression vector according to claim 14, which is plasmid pFC33.

16. A host which has been transformed with an expression vector as defined in claim 1 and in which thereby a protein of formula (I) as defined in claim 1 is capable of being expressed.

17. A host according to claim 16, wherein the host is a strain of E. coli.

18. A process for producing a protein which is capable of being detected by ELISA with an anti-human apoAI antiserum, which process comprises culturing a transformed host as defined in claim 16 and recovering the said protein thus obtained.

19. A protein which is capable of being detected

by ELISA with anti-human apoAI and which has the formula (1)

Met-X-Y (1)

wherein X is a bond, a carrier peptide sequence which comprises a sequence derived from the N-terminal amino acid residues of beta-galactosidase or a sequence comprising one or more IgG-binding domains of Protein A, or the pro sequence of human apoAI; and Y represents the sequence of human apoAI or a genetic variant thereof.

20. A protein according to claim 19, which is selected from Met-apoAI, Met-apoAI-T6, Met-apoAI-MI and Met-apoAI-T6/MI.

21. A protein according to claim 19, wherein the amino acid sequence Met-Thr-Met-Ile-Thr-Pro-Ser-Phe-Asp-Gly-Ser-Met is fused to an apolipoprotein selected from apoAI, apoAI-T6, apoAI-MI and apoAI-T6/MI.

22. A protein according to claim 19, wherein the amino acid sequence Met-Thr-Met-Ile-Thr-Asn-Ser-Arg-Gly-Ser-Met is fused to an apolipoprotein selected from apoAI, apoAI-T6, apoAI-MI and apoAI-T6/MI.

23. A protein according to claim 19, which is composed of (the first 11 amino acids of the CRO protein of phage lambda)-(248 amino acids of staphylococcal protein A (residues 23 to 270))-(Pro-Gly-Asp-Ser-Thr)-(the last 17 amino acids of beta-galactosdase)-(the 17 amino acids which contain a recognition sequence for the proteolytic enzyme enterokinase and which are Gly-Asp-Pro-Glu-Phe-Val-Asp-Asp-Asp-Asp-Lys-Ser-Ser-Arg-Gly-Ser-Met)-(apoAI, apoAI-T6, apoAI-MI or apoAI-T6/MI).

24. A protein according to claim 19, which is Met-proapoAI.

25. A pharmaceutical composition comprising a protein as defined in claim 19 as active ingredient and a pharmaceutically acceptable carrier or diluent.

Fig. 1.

APOA1

```
                                                    27                                                  54
GAT GAA CCC CCC CAG AGC CCC TGG GAT CGA GTG AAG GAC CTG GCC ACT GTG TAC
Asp Glu Pro Pro Gln Ser Pro Trp Asp Arg Val Lys Asp Leu Ala Thr Val Tyr

                                                    81                                                 108
GTG GAT GTG CTC AAA GAC AGC GGC AGA GAC TAT GTG TCC CAG TTT GAA GGC TCC
Val Asp Val Leu Lys Asp Ser Gly Arg Asp Tyr Val Ser Gln Phe Glu Gly Ser

                                                   135                                                 162
GCC TTG GGA AAA CAG CTA AAC CTA AAG CTC CTT GAC AAC TGG GAC AGC GTG ACC
Ala Leu Gly Lys Gln Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Val Thr

                                                   189                                                 216
TCC ACC TTC AGC AAG CTG CGC GAA CAG CTC GGC CCT GTG ACC CAG GAG TTC TGG
Ser Thr Phe Ser Lys Leu Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Ph  Trp

                                                   243                                                 270
GAT AAC CTG GAA AAG GAG ACA GAG GGC CTG AGG CAG GAG ATG AGC AAG GAT CTG
Asp Asn Leu Glu Lys Glu Thr Glu Gly Leu Arg Gln Glu MET Ser Lys Asp Leu

                                                   297                                                 324
GAG GAG GTG AAG GCC AAG GTG CAG CCC TAC CTG GAC GAC TTC CAG AAG AAG TGG
Glu Glu Val Lys Ala Lys Val Gln Pro Tyr Leu Asp Asp Phe Gln Lys Lys Trp

                                                   351                                                 378
CAG GAG GAG ATG GAG CTC TAC CGC CAG AAG GTG GAG CCG CTG CGC GCA GAG CTC
Gln Glu Glu MET Glu Leu Tyr Arg Gln Lys Val Glu Pro Leu Arg Ala Glu Leu

                                                   405                                                 432
CAA GAG GGC GCG CGC CAG AAG CTG CAC GAG CTG CAA GAG AAG CTG AGC CCA CTG
Gln Glu Gly Ala Arg Gln Lys Leu His Glu Leu Gln Glu Lys Leu Ser Pro Leu

                                                   459                                                 486
GGC GAG GAG ATG CGC GAC CGC GCG CGC GCC CAT GTG GAC GCG CTG CGC ACG CAT
Gly Glu Glu MET Arg Asp Arg Ala Arg Ala His Val Asp Ala Leu Arg Thr His

                                                   513                                                 540
CTG GCC CCC TAC AGC GAC GAG CTG CGC CAG CGC TTG GCC GCG CGC CTT GAG GCT
Leu Ala Pro Tyr Ser Asp Glu Leu Arg Gln Arg Leu Ala Ala Arg Leu Glu Ala

                                                   567                                                 594
CTC AAG GAG AAC GGC GGC GCC AGA CTG GCC GAG TAC CAC GCC AAG GCC ACC GAG
Leu Lys Glu Asn Gly Gly Ala Arg Leu Ala Glu Tyr His Ala Lys Ala Thr Glu

                                                   621                                                 648
CAT CTG AGC ACG CTC AGC GAG AAG GCC AAG CCC GCG CTC GAG GAC CTC CGC CAA
His Leu Ser Thr Leu Ser Glu Lys Ala Lys Pro Ala Leu Glu Asp Leu Arg Gln

                                                   675                                                 702
GGC CTG CTG CCC GTG CTG GAG AGC TTC AAG GTC AGC TTC CTG AGC GCT CTC GAG
Gly Leu Leu Pro Val Leu Glu Sir Phe Lys Val Ser Phe Leu Ser Ala Leu Glu

                                                   729
GAG TAC ACT AAG AAG CTC AAC ACC CAG TGA
Glu Tyr Thr Lys Lys Leu Asn Thr Gln
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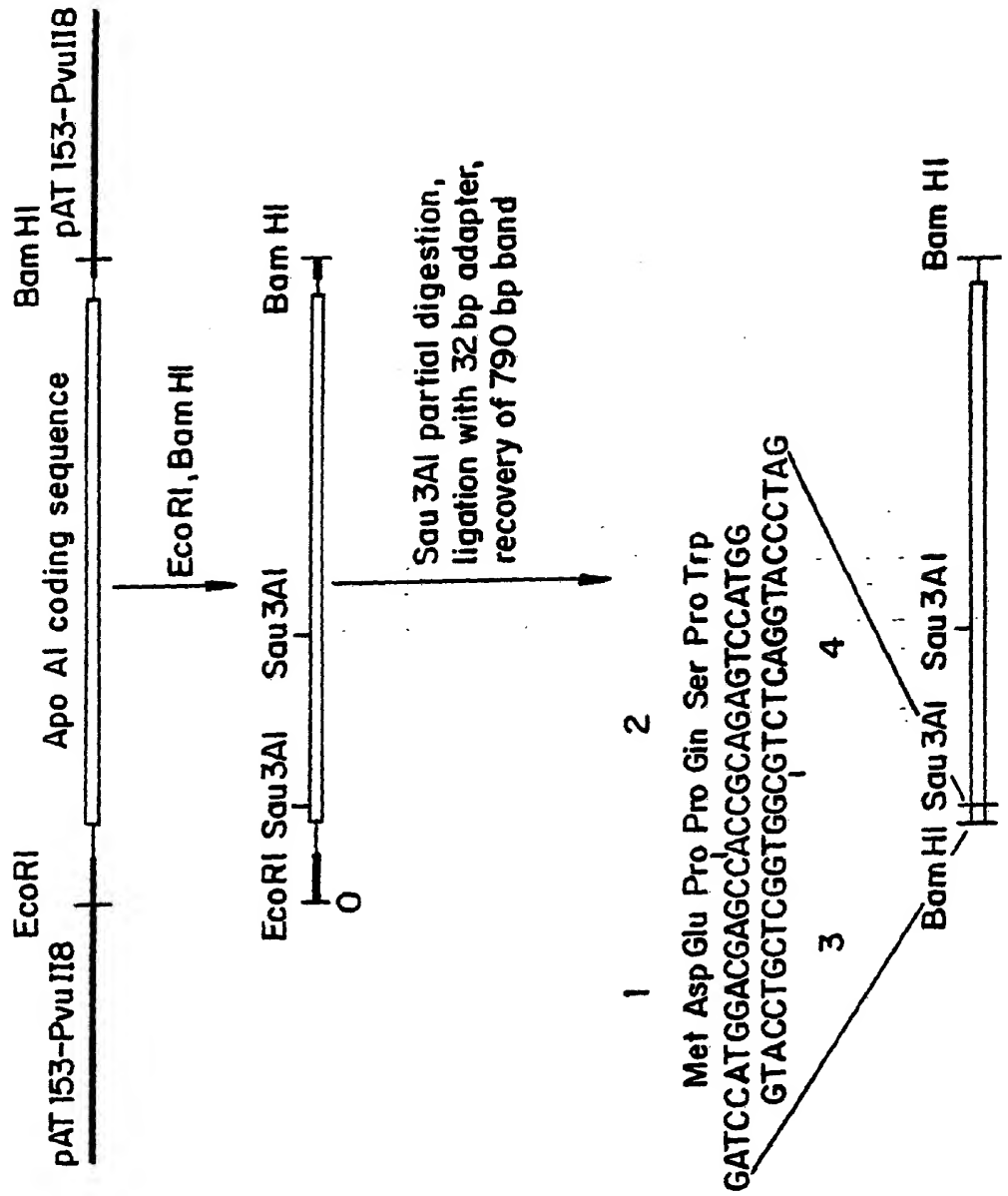
Fig. 2.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Apo AI coding sequence
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Bam HI
0
Sau 3AI partial digestion, ligation with 32 bp adapter, recovery of 790 bp band
1
2
3
4
Met Asp Glu Pro Pro Gln Ser Pro Trp
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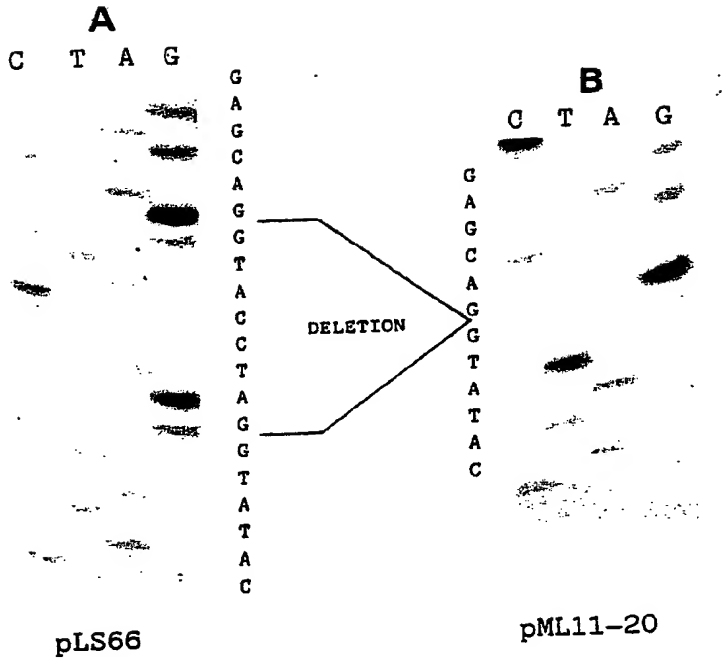
Sau3AI
Bam HI

Fig.3.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DELETION
B
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pML11-20

Fig.4.

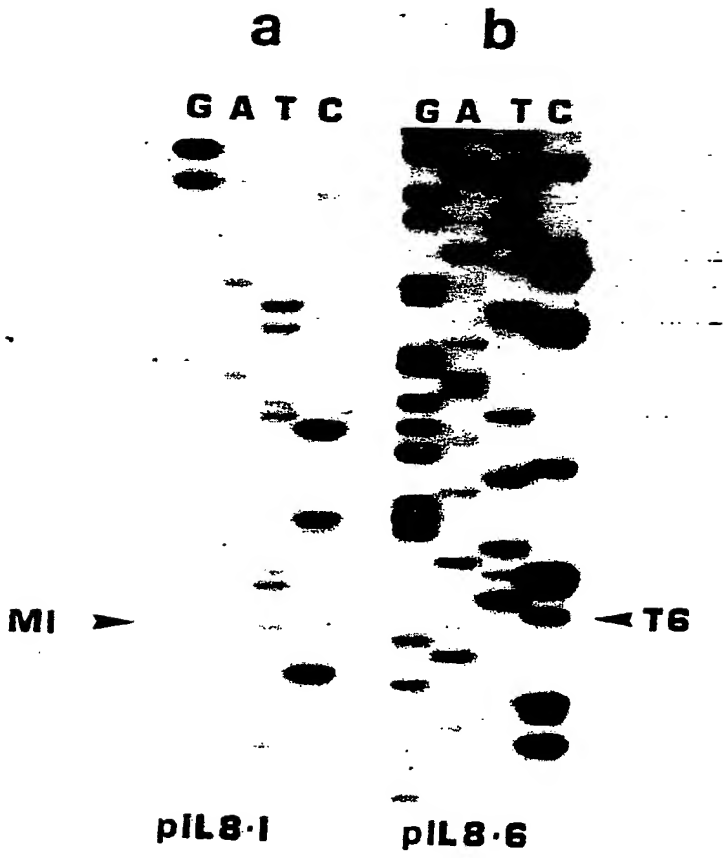


Fig. 5.

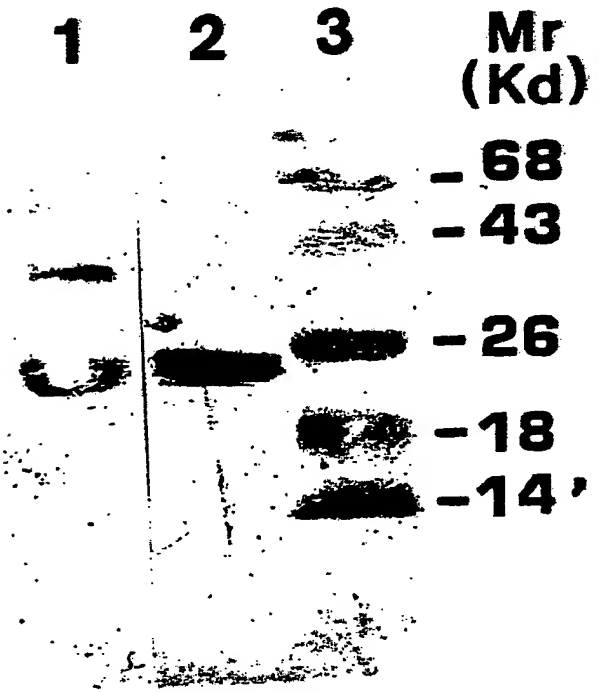
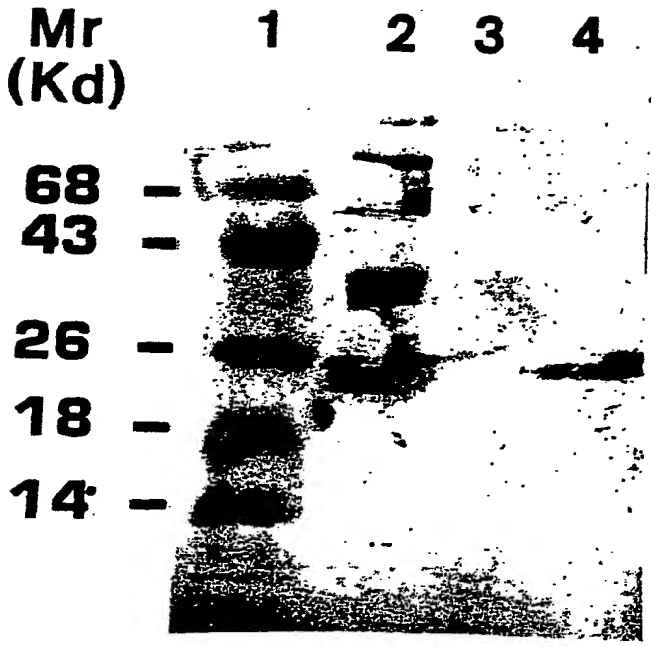
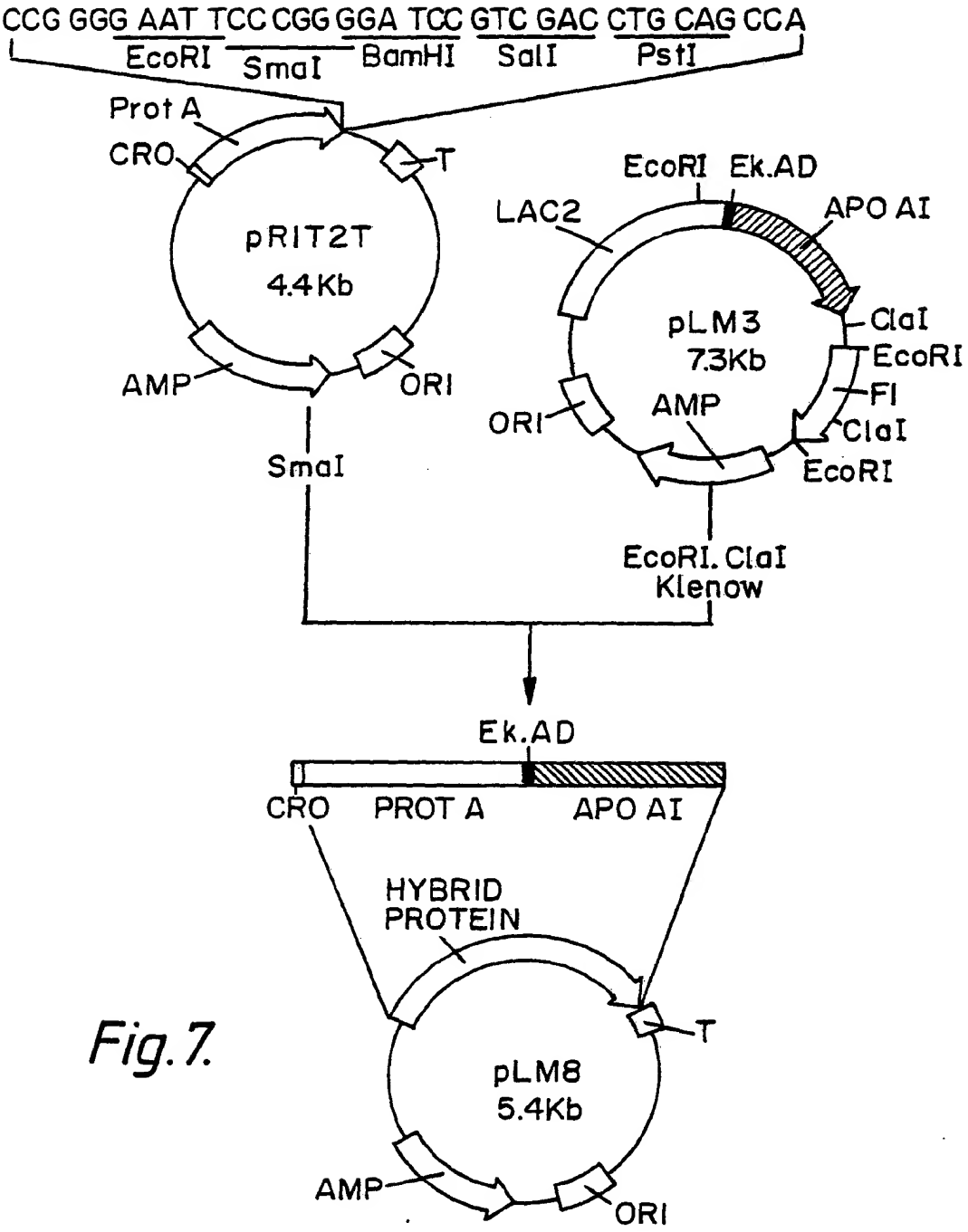


Fig. 6.

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SmaI
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Prot A
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4.4Kb
AMP
ORI
SmaI
EcoRI Ek.AD
LAC2
APO AI
pLM3
7.3Kb
ClaI
EcoRI
FI
ClaI
EcoRI
ORI
AMP
EcoRI. ClaI
Klenow
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T
pLM8
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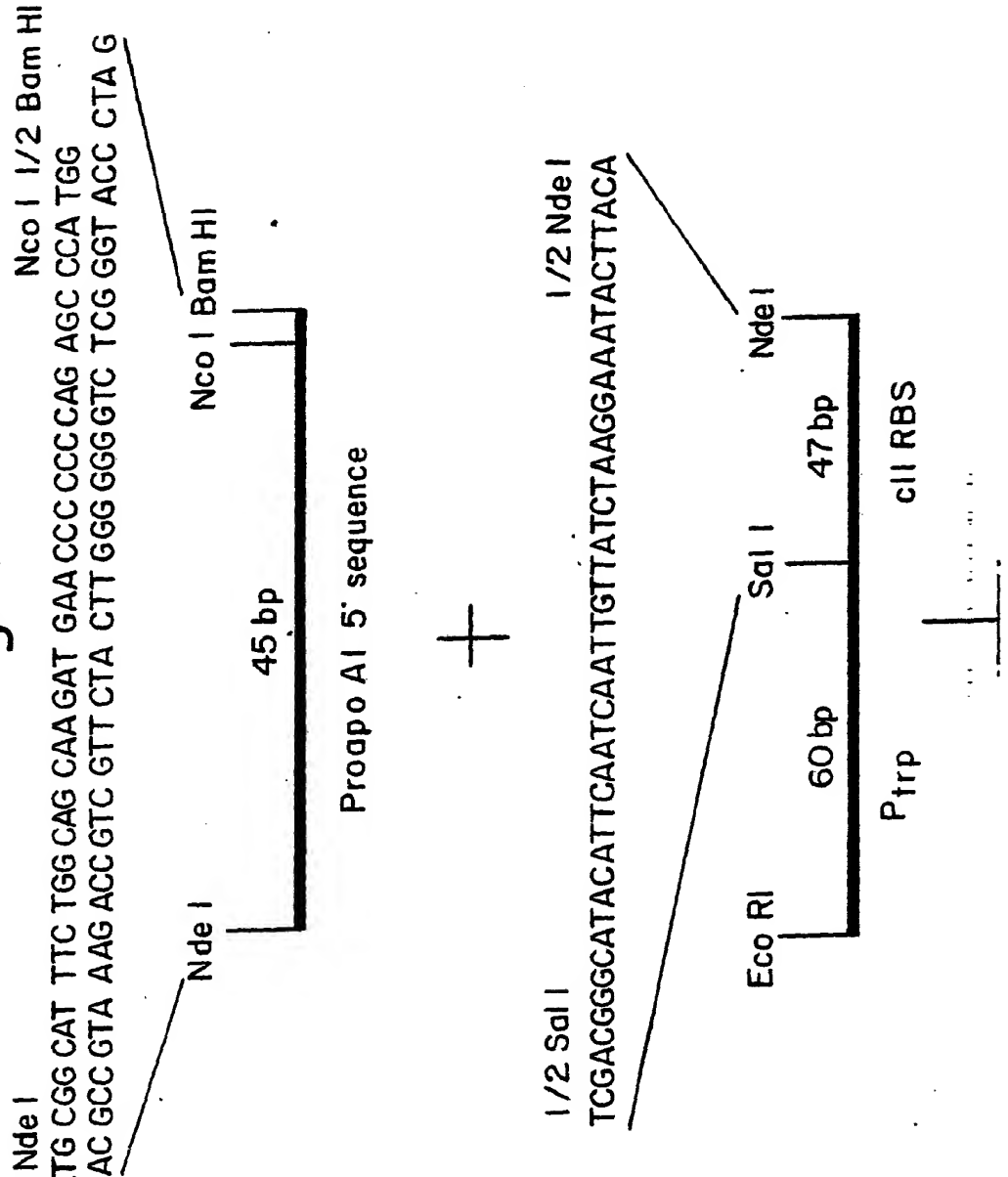
ORI

Fig. 7.

Fig. 8.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T ATG CGG CAT TTC TGG CAG CAA GAT GAA CCC CCC CAG AGC CCA TGG
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Nco I 1/2 Bam HI
Nde I
Nco I Bam HI
45 bp
Proapo AI 5' sequence
1/2 Sal I
TCGACGGGCATACATTCAATCAATTGTTATCTAAGGAAATACTTACA
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Eco RI
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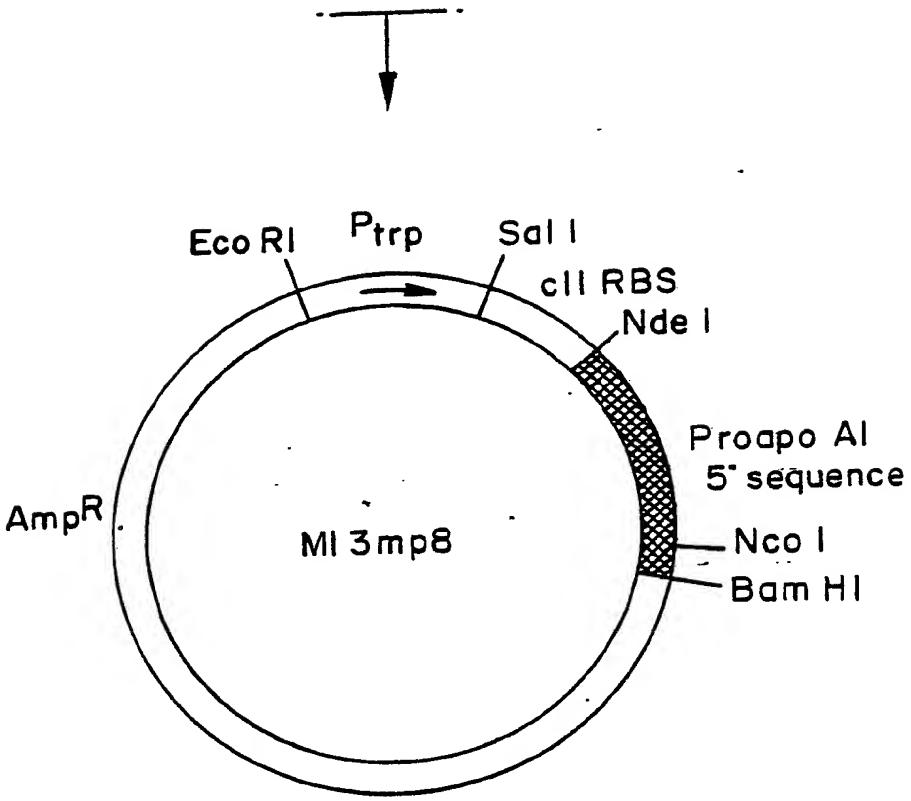
Nde I
60 bp
47 bp
Ptrp
cII RBS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Ptrp
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Nde I
Proapo AI
5' sequence
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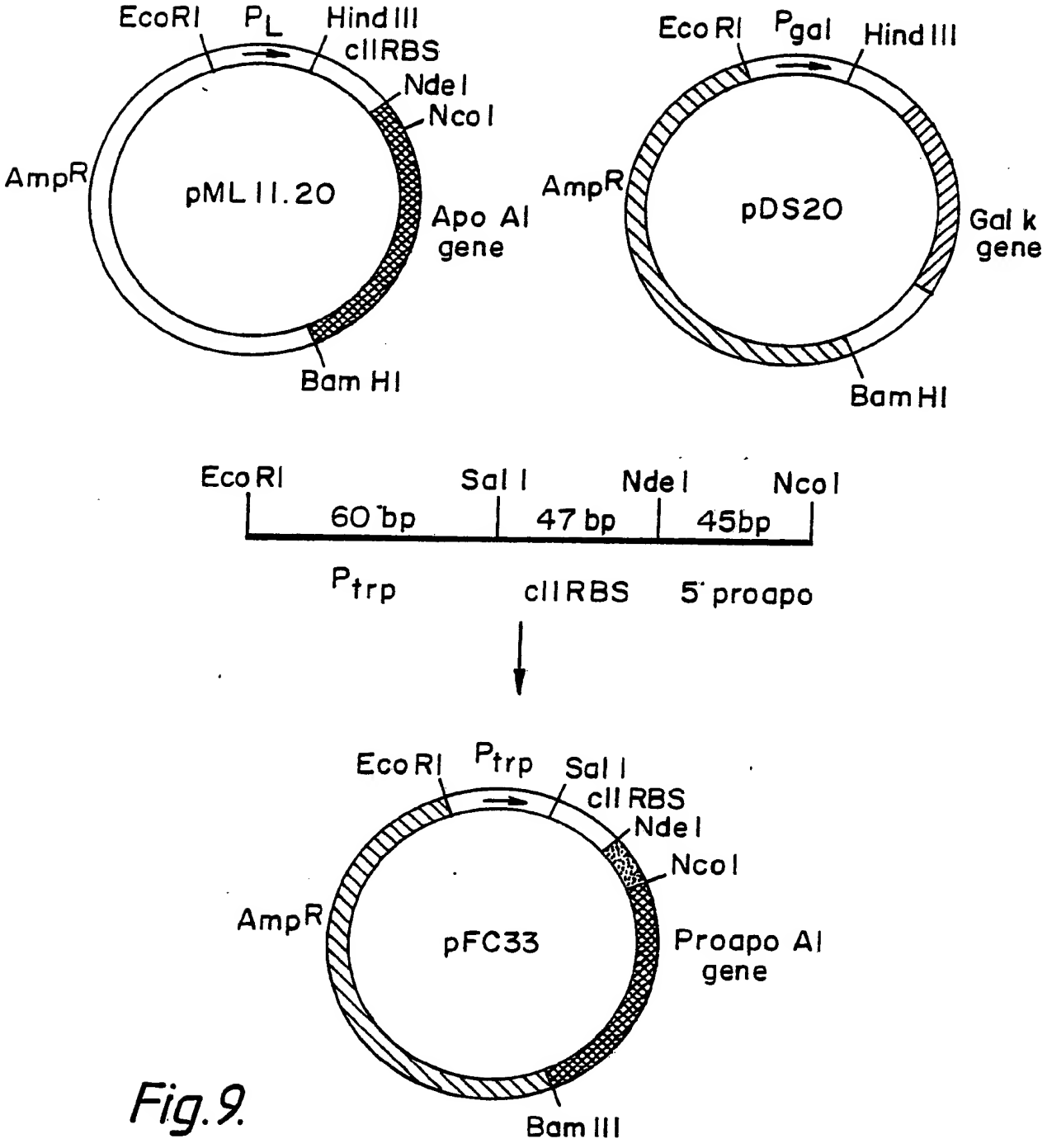
MI 3mp8

Fig.8(cont.)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BamHI
EcoRI
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AmpR
pDS20
Gal k gene
BamHI
EcoRI
SalI
NdeI
NcoI
60 bp
47 bp
45bp
Ptrp
cIIRBS
5' proapo
EcoRI
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NcoI
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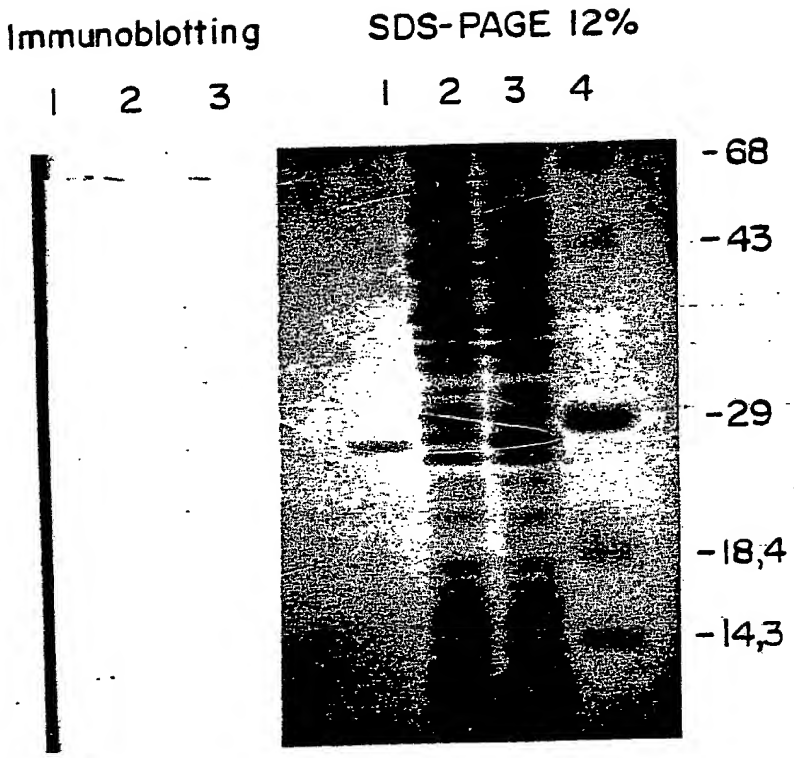
Proapo AI gene
BamIII

Fig.9.

Fig.10.



INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 87/00621

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; C 12 N 1/20; C 12 P 21/02; C 07 K 13/00; A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	FEBS Letters, volume 194, no. 2, January 1986, Elsevier Science Publishers B.V. (Biomedical Division), pages 343-346 R. Lorenzetti et al.: "Expression of the human apolipoprotein AI gene fused to E. coli gene for β-galactosidase", pages 343-346 --	1-20,24, 25
X	WO, A, 86/04920 (BIOTECHNOLOGY RESEARCH PARTNERS LTD) 28 August 1986 see page 39, lines 10-15 --	1,2,16-20, 24,25
A	Patent Abstracts of Japan, volume 10, no. 276 (C-373)(2332), 19 September 1986, & JP, A, 6196998 (MITSUBISHI CHEM. IND. LTD) 15 May 1986 --	1-25
T	The EMBO Journal, volume 6, no. 11, November 1987, IRL Press Ltd, (Oxford, GB), L. Monaco et al.: "A recombinant apoA-1-protein A hybrid reproduces the binding parameters of HDL to its receptor", pages 3253-3260, see the whole article	1-25

* Special categories of cited documents: [10]

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29th January 1988	17 MAR 1988
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 8700621 SA 19324

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 01/03/88 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8604920	28-08-86	AU-A- 5513586	10-09-86
		EP-A- 0217822	15-04-87
		JP-T- 62501957	06-08-87